

ORIGINAL RESEARCH



## Metabolic enzymes expressed by cancer cells impact the immune infiltrate

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### ABSTRACT

The expression of two metabolic enzymes, *i.e.*, aldehyde dehydrogenase 7 family, member A1 (ALDH7A1) and lipase C, hepatic type (LIPC) by malignant cells, has been measured by immunohistochemical methods in non-small cell lung carcinoma (NSCLC) biopsies, and has been attributed negative and positive prognostic value, respectively. Here, we demonstrate that the protein levels of ALDH7A1 and LIPC correlate with the levels of the corresponding mRNAs. Bioinformatic analyses of gene expression data from 4921 cancer patients revealed that the expression of LIPC positively correlates with abundant tumor infiltration by myeloid and lymphoid cells in NSCLC, breast carcinoma, colorectal cancer and melanoma samples. In contrast, high levels of ALDH7A1 were associated with a paucity of immune effectors within the tumor bed. These data reinforce the notion that the metabolism of cancer cells has a major impact on immune and inflammatory processes in the tumor microenvironment, pointing to hitherto unsuspected intersections between oncometabolism and immunometabolism.

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## Introduction

The metabolism of malignant cells is rewired towards anabolic reactions that facilitate proliferation, resistance against a hostile milieu, and immune escape.<sup>1–5</sup> Although most of the literature on oncometabolism has been elaborated based on *in vitro* studies, in which malignant cells are usually cultured as cell lines (*i.e.*, in the absence of additional cellular and non-cellular components of the tumor microenvironment), it has become clear that tumors function like a microecosystem, in which different cell types interact to favor tumor insurgence and progression.<sup>6,7</sup> Moreover, the metabolism of immune cells is largely influenced by the tumor milieu, often skewing the differentiation of myeloid and lymphoid cell types towards a pro-inflammatory and immunosuppressive phenotype.<sup>8–10</sup>

Hence, oncometabolism and immunometabolism are connected by a bidirectional and intimate relationship that has profound implications for targeting cancer with metabolic interventions. As a standalone example, fasting, *i.e.*, the temporary reduction of caloric intake, can limit tumor growth *in vitro*, as well as *in vivo*, in human cancers xenografted into immunodeficient mice.<sup>11</sup> The effect of such treatments depends to some extent on the intrinsic characteristic of tumor cells including their dependency on glycolysis or glutaminolysis.<sup>12–14</sup> In addition, fasting can stimulate

anticancer immune responses, and this effect is likely to stem from the activation of autophagy in cancer cells, favoring their immune recognition,<sup>15,16</sup> as well as from direct stimulatory effects on immune cells.<sup>17,18</sup> Similarly, inhibition of glycolysis has been suggested to directly affect tumor growth, yet also stimulates anticancer immune responses.<sup>19</sup>

Driven by these considerations, we decided to evaluate the impact of specific metabolic enzymes on the immune infiltrate of non-small cell lung carcinoma (NSCLC) lesions, the prognosis of which is known to be dictated by anticancer immune responses.<sup>20–28</sup> Based on an admittedly cell-autonomous perspective of NSCLC, we previously reported that the expression or activity enzymes involved in (1) vitamin B6 metabolism, such as pyridoxal kinase (PDXK) and aldehyde dehydrogenase 7 family, member A1, (ALDH7A1), (2) lipid synthesis, such as lipase C, hepatic type (LIPC), or (3) poly-ADP ribosylation, such as poly(ADP-ribose) polymerase 1 (PARP1) would affect the response of NSCLC cells to chemotherapy *in vitro* and *in vivo*, and affect patient prognosis.<sup>29–35</sup> However, we have not yet explored the impact of these enzymes on anticancer immune responses. Here, we investigated the possibility that such enzymes might affect natural or therapy-driven anticancer immunosurveillance using a bioinformatic approach.

## Results

### Correlations of mRNA and protein expression levels for ALDH7A1 and LIPC

The notion that LIPC, PARP1 and PDXK expression affects the response of NSCLCs to therapy and the prognosis of NSCLC patients has initially been acquired by studying the CHEMORES cohort ([www.chemores.ki.se](http://www.chemores.ki.se)), followed by validation in additional patient series. In particular, high LIPC expression (as measured by immunohistochemistry), low PARP1 activity (as measured by assessing the abundance of the PARP1 product poly(ADP-ribose) using immunochemistry) and high PDXK levels (as measured by immunohistochemistry) in NSCLC cells were identified as favorable prognostic biomarkers for NSCLC patients.<sup>29–33,35</sup> For ALDH7A1, we did observe an effect on cisplatin responses *in vitro*, yet we did not find a significant impact on progression-free survival or overall survival in the CHEMORES cohort,<sup>29</sup> contrasting with a more recent report demonstrating that high ALDH7A1 expression is associated with recurrence in patients with surgically resected NSCLC.<sup>36</sup> Since mRNA expression data (as determined by transcriptomic analyses) in biopsies from cancer patients are considerably more abundant than immunohistochemical data, we reasoned that a meta-analysis of the impact of such enzymes on immune infiltration should be based on mRNA rather than protein levels. To this aim, we determined the correlation between mRNA and protein levels for a number of gene products within the CHEMORES database. Positive correlations between mRNA levels (as determined by transcriptomic analysis) and protein levels (as determined by immunohistochemistry) were observed for ALDH7A1, BCL2L1 (better known as BCL-X<sub>L</sub>)<sup>29</sup> and LIPC, but not for PARP1, PARP2, pyridoxal phosphatase (PDXP) and other proteins such as ALK receptor tyrosine kinase (ALK) and WD repeat and SOCS box containing 2 (WSB2) (Figure 1A–D). We also determined the correlation between the densities of tumor-infiltrating CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs), FOXP3<sup>+</sup> regulatory T (T<sub>REG</sub>) cells, and DC-LAMP<sup>+</sup> dendritic cells (DCs) as determined by immunohistochemistry, and the abundance of different immune subsets measured with the Microenvironment Cell Populations-counter (MCP-counter) method, which is based on the expression levels of signature mRNAs.<sup>37</sup> Convincing correlations were observed for CD8 and CTLs, but neither for FOXP3 and T<sub>REG</sub> cells, nor for DC-LAMP and DCs (Figure 1E). Driven by these results, we decided to focus our study on ALDH7A1 and LIPC in relationship to the tumor immune infiltrate.

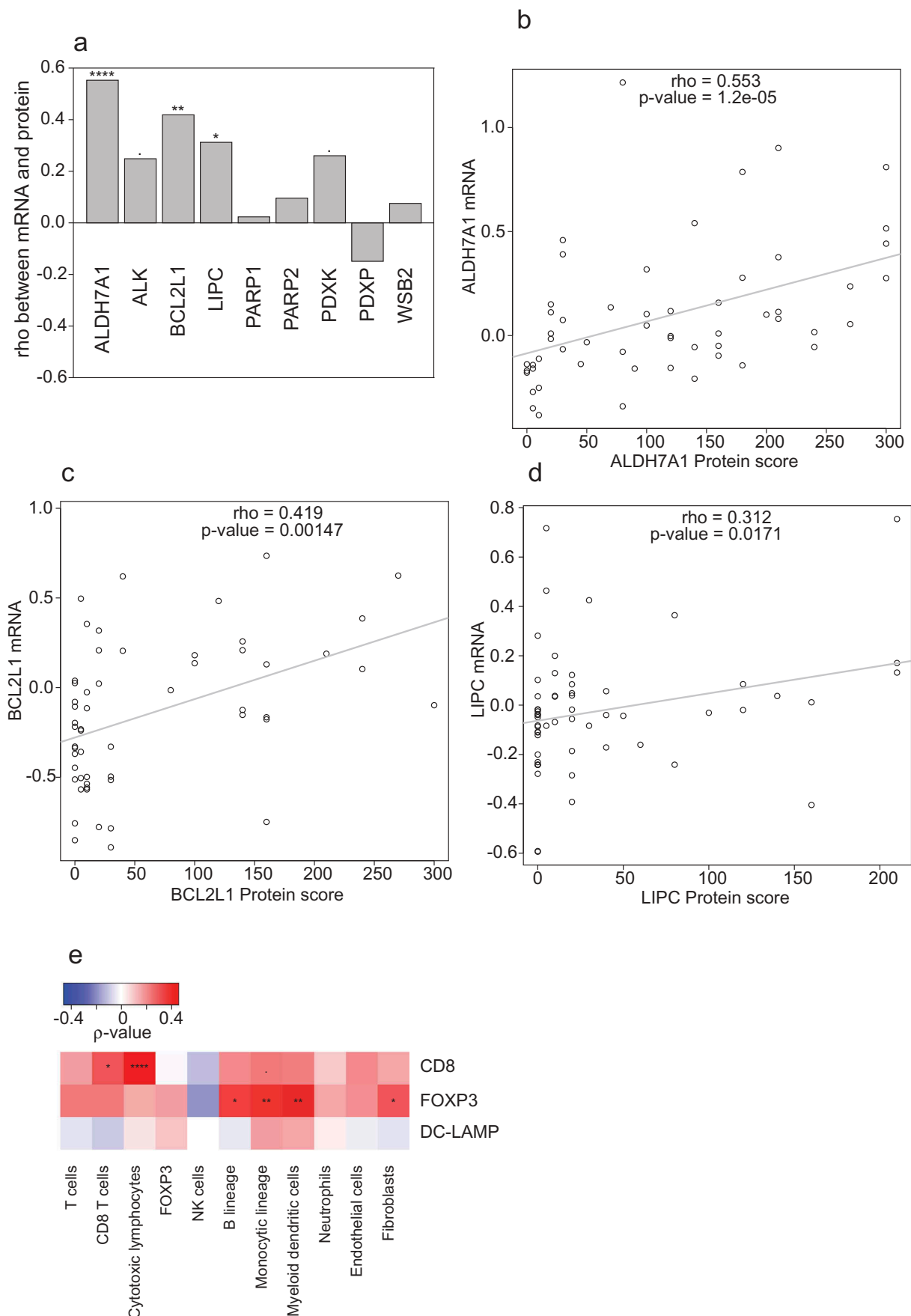
### Associations between immunosurveillance and the expression of ALDH7A1, BCL2L1 and LIPC and across multiple cancers

In samples from the CHEMORES cohort, the protein levels of BCL2L1 and LIPC correlated with a prevalence of DCs as determined by the MCP counter, while ALDH7A1 levels exhibited a trend towards negative correlation with the abundance of NK cells (Figure 2A). These correlations were much more pronounced when only mRNA levels were analyzed. ALDH7A1 mRNA levels negatively correlated with those of

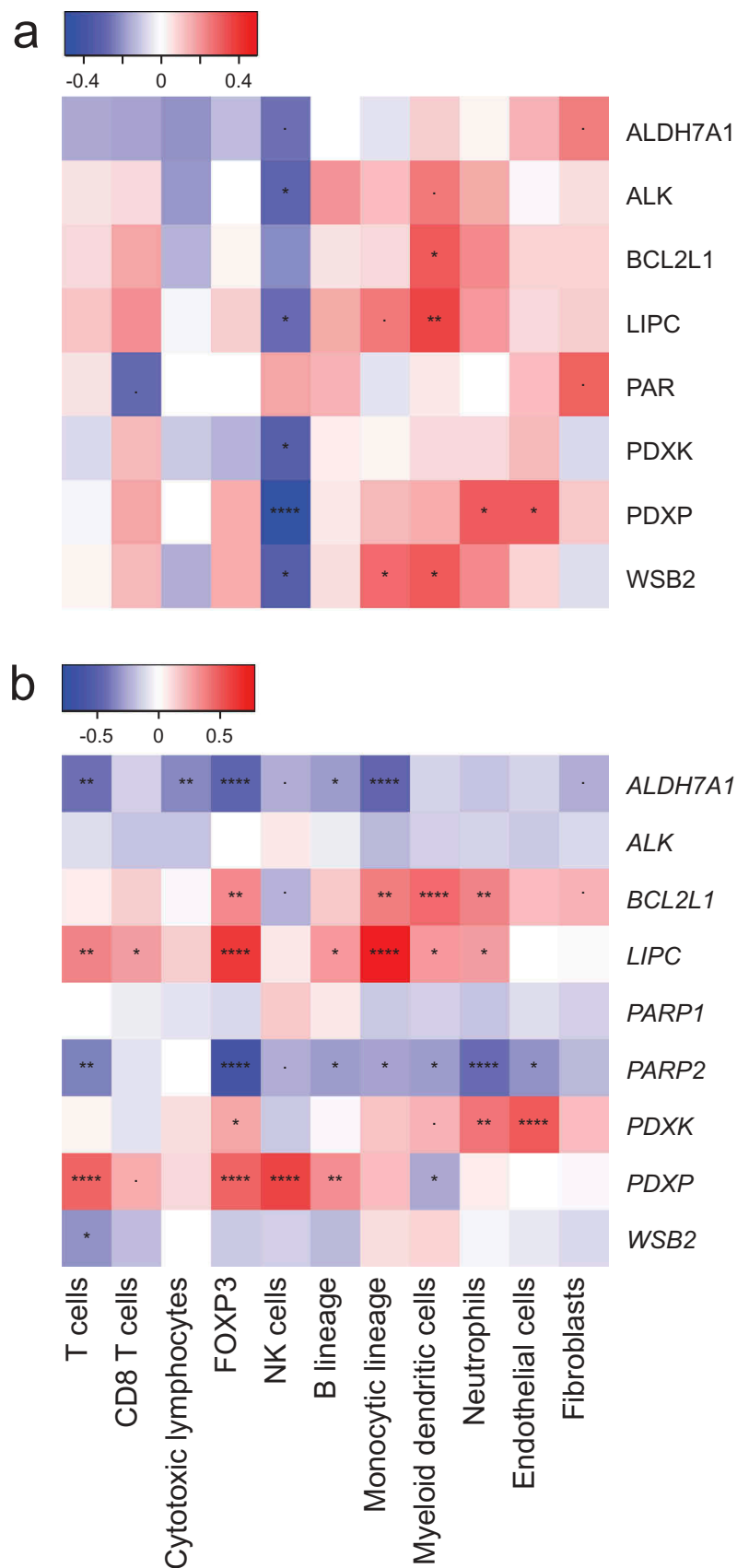
multiple immune effectors (T cells, CTLs, T<sub>REG</sub> cells, B cells and monocytic cells) (Figure 2B). BCL2L1 mRNA levels correlated with the abundance of the FOXP3 mRNA, as well as with that of mRNAs from myeloid cell subsets (cells from the monocytic lineage, myeloid DCs, neutrophils). Similarly, the levels of LIPC mRNA positively correlated with those characterizing a number of distinct immune subset (T cells, CD8<sup>+</sup> T cells, T<sub>REG</sub> cells, B cells, cells from the monocytic lineage, myeloid DCs and neutrophils).

The positive correlation between LIPC levels and immune effectors could not be attributed to the preferential expression of BCL2L1 or LIPC by leukocytes. Indeed, BCL2L1 and LIPC were typically expressed by NSCLC cells, not by tumor-infiltrating immune cells (Figure 3). Moreover, BCL2L1 and LIPC mRNA levels are not particularly high in leukemic cells or normal leukocytes subpopulations, as compared to a negative control mRNA (TYR, which codes for the melanoma-specific protein tyrosinase) and a positive control mRNA (CD45, encoding a common leukocyte antigen) (Figure 4). Based on these considerations, it appears plausible that it is indeed the expression of LIPC and ALDH7A1 by malignant cells (as opposed to stromal elements) that exhibits a positive and negative correlation, respectively, with the immune infiltrate.

We next investigated the correlation between ALDH7A1, BCL2L1 and LIPC mRNA expression levels and the abundance of an array of immune cell subsets using the MCP-counter. To this aim, we generated a database encompassing gene expression data from 4921 patients with NSCLC, breast cancer, colorectal carcinoma or melanoma (Table 1). The correlations were calculated separately for each cohort of patients (5 cohorts with NSCLC, 6 with breast cancer, 3 with colorectal carcinoma, 5 with melanoma) and then for each cancer type together. ALDH7A1 mRNA levels were associated with a paucity of immune cells and fibroblasts, in particular amongst NSCLC and breast cancer patients (Figure 5A). Similarly, BCL2L1 mRNA levels tended to correlate with limited infiltration by various immune subsets, in particular CTLs and NK cells, in NSCLC, breast and colon cancer, but not in melanoma (Figure 5B). The most spectacular associations were found for LIPC. The levels of LIPC mRNA were associated with a poor immune infiltrate and even a scarcity of other non-malignant cell types such as endothelial cells and/or fibroblasts in all cancer types analyzed in this study (Figure 5C). This does not seem to be related to stem cells, as LIPC (nor ALDH7A1) did correlate with the stem cell marker PROM1 (CD133) (see sheet 1 of Supplemental Table S1). From these results, we conclude that LIPC and ALDH7A1 constitute two cancer-relevant metabolic enzymes whose expression correlates with the immune infiltrate in an opposite fashion. Notwithstanding the strong association between LIPC and ALDH7A1 mRNA levels and the immune infiltrate, there was little or no correlation between their abundance and patient survival across multiple cancer types (Suppl. Fig. S1–S4). This might reflect the possibility that ALDH7A1 and LIPC effects on cancer prognosis cancel each other because of their ambiguous impact on the immune infiltrate (which involves both positive and regulatory elements) and tumor cell-intrinsic

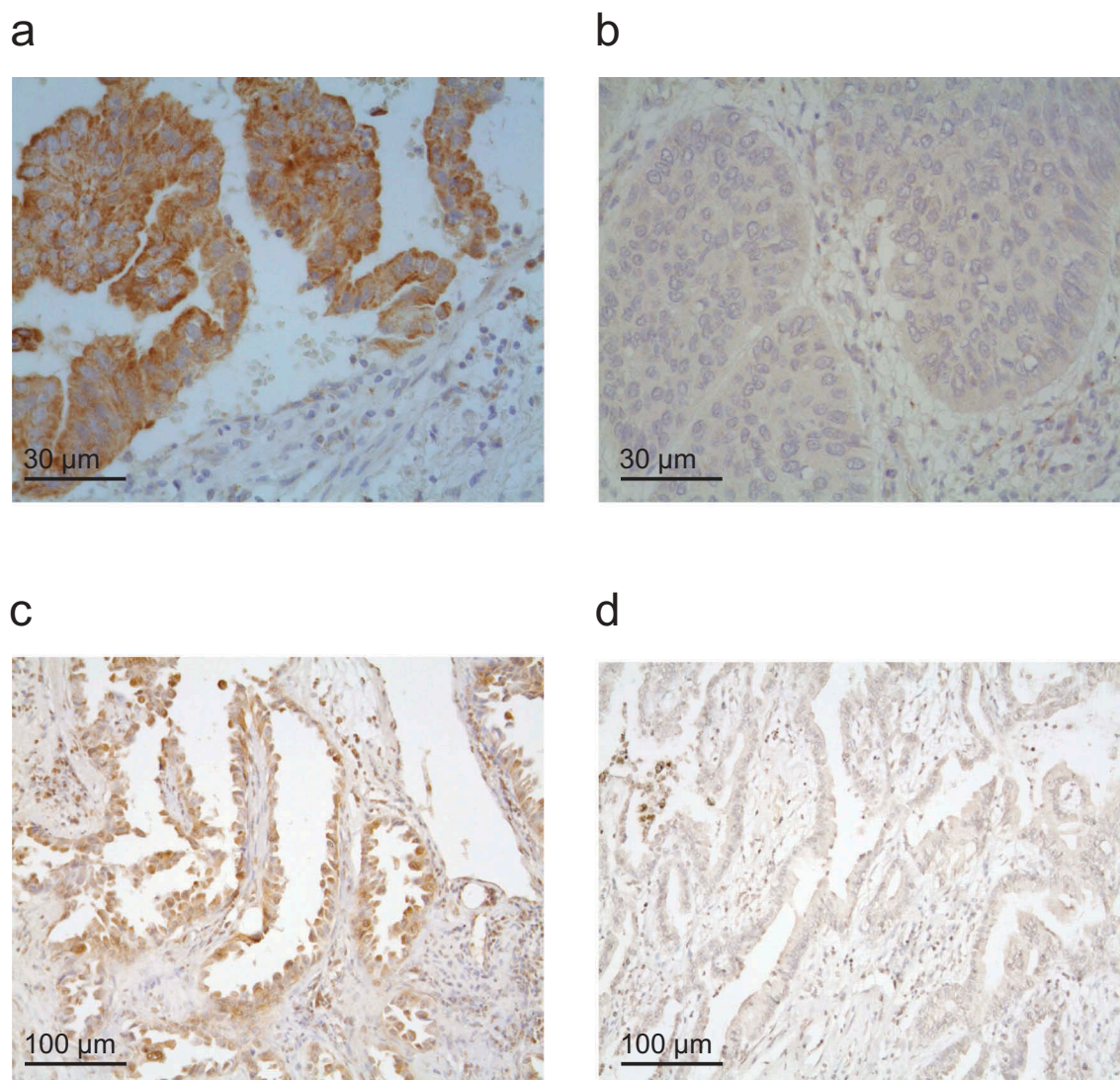


**Figure 1.** (a): Spearman's correlation coefficients between mRNA levels (as determined by transcriptomic studies) and protein score (as determined by immunohistochemistry). Significant correlations are annotated: \* $p < 0.1$ , \*\* $p < 0.05$ , \*\*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ ). (b,c): Scatter plot representations of protein scores *versus* mRNA levels for significant correlations from panel A (ALDH7A1, BCL2L1 and LIPC). Significant correlations are annotated: \* $p < 0.1$ , \*\* $p < 0.05$ , \*\*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ ). (e): Spearman's correlation coefficients between immune infiltrate estimated by microarray expression deconvolution (MCP-counter method) and protein score estimated from immunofluorescence).



**Figure 2.** (a,b): Spearman's correlations coefficients between immune infiltrate estimates (MCP-counter method) and protein score (estimated from immunofluorescence, (a)) or mRNA levels (as determined by transcriptomic studies, (b)). Significant correlations are annotated: \* $p < 0.1$ , \*\* $p < 0.05$ , \*\*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .





**Figure 3.** (a–d): Representative images for BCL2L1 (a,b) and LIPC (c,d) immunohistochemistry in NSCLC samples with high (a,c) or low (b,d) expression levels.

characteristics. Moreover, neither *LIPC* nor *ALDH7A1* correlated with the mRNA levels of *PD-1*, which codes for an important immune checkpoint molecule relevant to NSCLC, or *LKB1*, which codes for a metabolic regulator with relevance to NSCLC (sheet 2,3 of Supplemental Table S1).

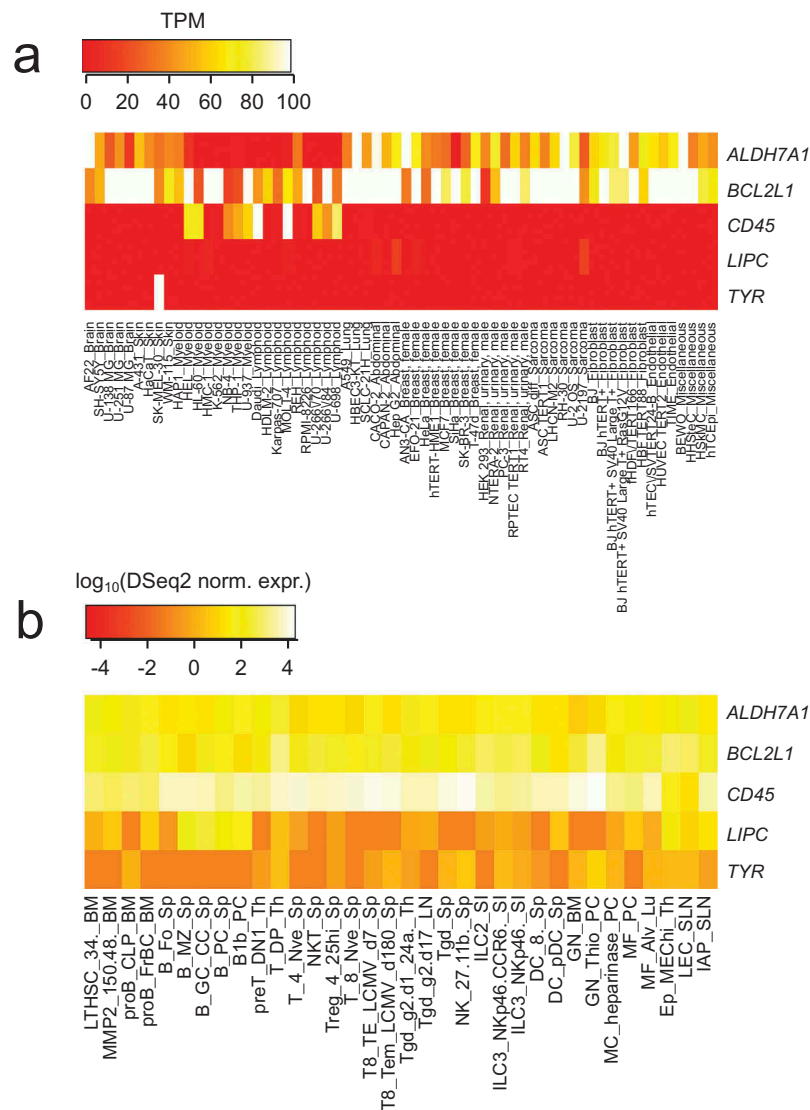
## Discussion

*ALDH7A1* is an aldehyde dehydrogenase with an important role in the detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation. *ALDH7A1* is best known for the fact that loss-of-function mutations of this gene cause pyridoxine-dependent epilepsy.<sup>38</sup> Moreover, knockdown of *ALDH7A1* sensitizes NSCLC cells to the lethal effect of cisplatin.<sup>29</sup> Interesting, *ALDH7A1* is overexpressed in cancer stem cells<sup>39</sup> and is druggable.<sup>40</sup> However, to the best of our knowledge, the possibility to inhibit *ALDH7A1* to obtain antineoplastic effects has not been investigated yet in preclinical models. In one study of *ALDH7A1* expression in NSCLC patients subjected to surgery, low *ALDH7A1* levels were accompanied by improved recurrence-free and overall survival.<sup>36</sup> Mechanistically, this may be

linked to improved anticancer immunosurveillance, given that *ALDH7A1*<sup>high</sup> tumors are immunologically desert. However, how high levels of *ALDH7A1* might condition the tumor microenvironment to cause a general paucity of immune effectors remains to be determined.

*BCL2L1* is well known for its cell-autonomous apoptosis- and autophagy-inhibitory effects on cancer cells,<sup>41,42</sup> and is well possible that these features indirectly impact on the tumor microenvironment. Although *BCL2L1* was found to positively correlate with the myeloid immune infiltrate in our exploratory study (on the CHEMORES cohort), the correlations turned out to be mostly negative (in particular within the NK cell and T cell compartments) across several NSCLC, breast cancer and colon cancer cohorts. How *BCL2L1* expression (mostly by neoplastic cells) can mediate such effects remains to be studied.

*LIPC* catalyzes the hydrolysis of triacylglycerides, and is mostly known for its participation in the release of free fatty acids from intermediate-density lipoproteins, thus generating low-density lipoproteins.<sup>43</sup> Genetic variants of *LIPC* affect dyslipidemia, for instance in the context of coronary artery disease.<sup>44,45</sup> The function of *LIPC* in cancer is unknown. The



**Figure 4.** (a): mRNA abundance in Transcripts Per Million (TPM, as provided by The Human Protein Atlas website), for *ALDH7A1*, *BCL2L1*, *CD45*, *LIPC* and *TYR* in different cancer cell lines. TPM is artificially limited to an upper value of 100, for improved visualization. (b): mRNA normalized expression levels, in  $\log^{10}$  scale, estimated by the DESQ2 package on RNA-seq data (as provided by the “Immunological Genome Project” website), for *ALDH7A1*, *BCL2L1*, *CD45*, *LIPC* and *TYR* in different immune cell types.

only study dealing with the impact of LIPC on cancer prognosis demonstrated that high LIPC protein levels correlate with improved progression-free and overall survival in two distinct NSCLC patient cohorts<sup>46</sup> (and TCGA consortium). Initially, we identified LIPC as a factor involved in cisplatin-resistance in multiple different human NSCLC cell line.<sup>31</sup> This finding is apparently at odds with the fact that low LIPC expression is a poor prognostic marker, yet such stage I NSCLC patients might benefit from a platinum based chemotherapy as LIPC<sup>low</sup> patients receiving adjuvant chemotherapy exhibited an OS that was comparable to that of LIPC<sup>high</sup> patients, irrespective of their status of therapy receiver.<sup>29</sup> The data reported here suggest yet another possibility, namely that elevated LIPC expression by malignant cells modulates the tumor microenvironment to improve anticancer immunosurveillance. However, it is not possible to establish causal relationships at this point. Moreover, the potential mechanisms

through which LIPC would favor anticancer immune responses (rather than pro-carcinogenic inflammatory processes) remain entirely obscure.

The meta-analysis of different cancer types that we performed suggests that an elevated expression of LIPC or reduced ALDH7A1 levels similarly correlate with an increased presence of multiple distinct immune cell subtypes, some of which are involved in anticancer immunosurveillance (such as DCs and CTLs) while others are rather immunosuppressive (such as T<sub>REG</sub> cells and granulocytes).<sup>24</sup> In this context, it may be important to note that immunologically “cold” tumors usually lack both immunostimulatory and suppressive cell types altogether, while “hot” tumors contain a high frequency of different immune cells subtypes because of the intrinsic organization of the immune infiltrate that is particularly elaborate in breast cancer.<sup>47–53</sup> Thus, LIPC and ALDH7A1 might have an influence on the overall density of the leukocyte

**Table 1.** List of databases analyzed in this paper.

Cancer type	Cohort name	Number of samples	Characteristics of the cohort	Reference	Platform
Melanoma	Xu	83	Primary and metastatic tumors	GSE8401	Affymetrix Human Genome U133A Array
Melanoma	Harlin	44	Metastatic tumors	GSE12627	Affymetrix Human Genome U133A Array
Melanoma	Bogunovic	44	Metastatic tumors	GSE19234	Affymetrix Human Genome U133 Plus 2.0 Array
Melanoma	RikerMel	56	Primary and metastatic tumors	GSE7553	Affymetrix Human Genome U133 Plus 2.0 Array
Melanoma	Talantov	45	Primary tumors	GSE3189	Affymetrix Human Genome U133A Array
Colon	BittColon	307	Various colon tumors	GSE2109	Affymetrix Human Genome U95 Version 2 Array
Colorectal	Smith	177	Various colorectal tumors	GSE17536	Affymetrix Human Genome U133 Plus 2.0 Array
Colon	TCGA	174	Various colon tumors	TCGA consortium	Agilent 244K Custom Gene Expression G4502A-07-3
Breast	METABRIC	1781	Various breast tumors	METABRIC Study	Illumina HT-12 v3
Breast	TCGA	522	Various breast tumors	TCGA consortium	Agilent 244K Custom Gene Expression G4502A-07-1
Breast	Bonnefoi	161	Locally advance or large operable breast tumors, estrogen receptor negative	GSE6861	Affymetrix Human X3P Array
Breast	Hatzis	198	HER2 negative breast tumors	GSE25065	Affymetrix Human Genome U133A Array
Breast	Tabchy	178	Various type of breast tumors before treatment	GSE20271	Affymetrix Human Genome U133A Array
Breast	Korde	61	Various type of breast tumors, stage 2 or 3 breast cancer with tumor size $\geq 2$ cm at patients selection, prior to AC treatment	GSE18728	Affymetrix Human Genome U133 Plus 2.0 Array
Lung	AdenoConsortium	462	Various type of Adenocarcinomas	Director's Challenge Lung Study, National Cancer Institute (NHI)	Affymetrix Human Genome U133A Array
Lung	Lee	138	Adenocarcinoma and squamous cell carcinoma	GSE8894	Affymetrix Human Genome U133 Plus 2.0 Array
Lung	Okayama	226	Adenocarcinoma	GSE31210	Affymetrix Human Genome U133 Plus 2.0 Array
Lung	Raponi	130	Squamous cell carcinoma	GSE4573	Affymetrix Human Genome U133A Array
Lung	TCGA	134	Squamous cell carcinoma	TCGA consortium	Affymetrix Human Genome U133A Array

infiltrate, while other factors may affect the proportion and functional interactions among positive and negative elements of local immune circuitries.<sup>54</sup>

In sum, our work illustrates a potentially important crosstalk between oncometabolism and immunometabolism that warrants further mechanistic and preclinical evaluation.

## Material and methods

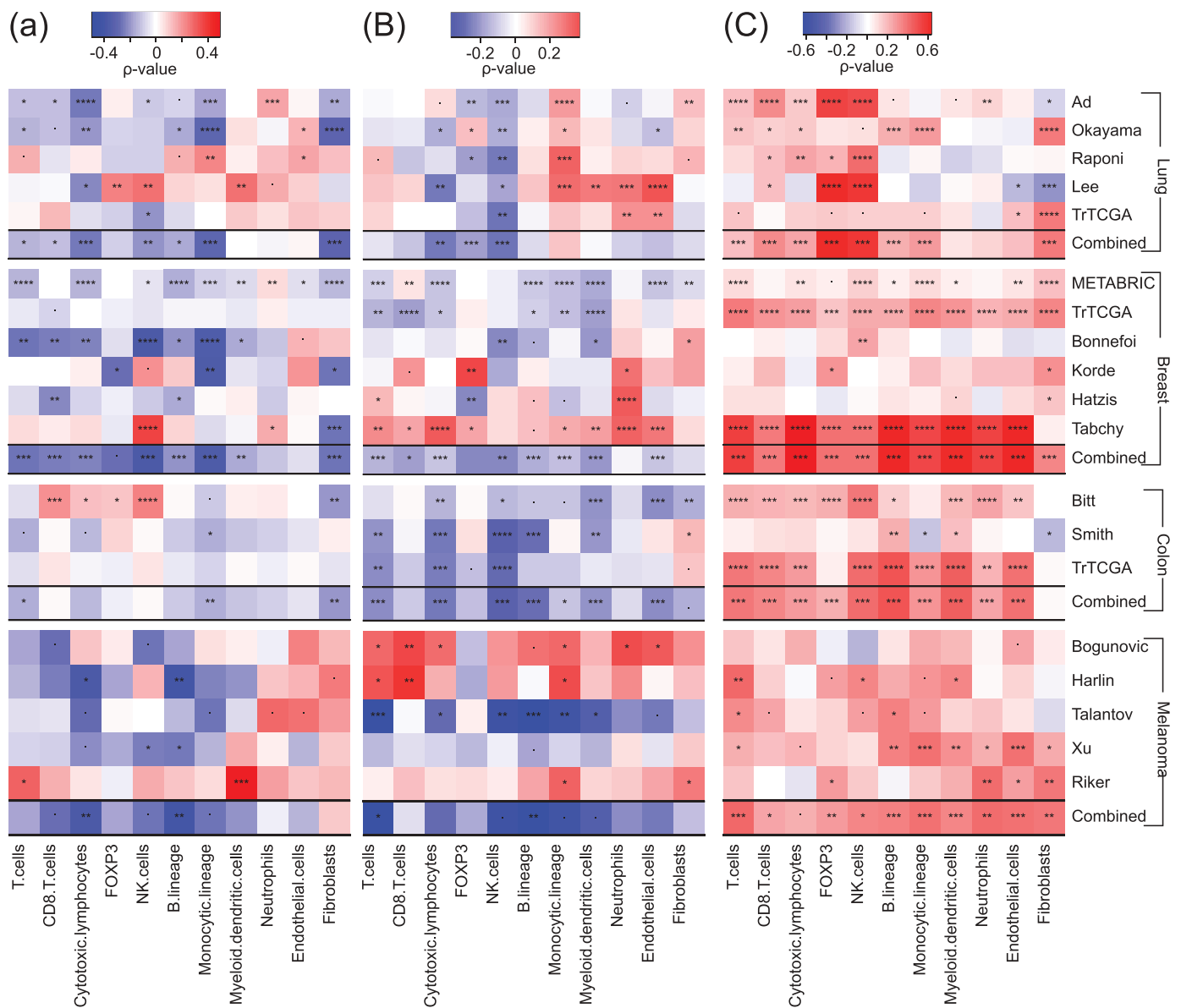
### Data from CHEMORES

We used 58 NSCLC biopsies from the CHEMORES cohort (<http://www.chemores.ki.se>), for which transcriptomic data were available and protein scores for ALDH7A1, ALK, BCL2L1, LIPC, poly(ADP)-ribose, PDXK, PDXP and WSB2 were estimated by immunohistochemistry. We were able to estimate the immune infiltration in 51 of 58 samples, using CD8, FOXP3 and DC-LAMP as immunohistochemical biomarkers.

### Immunohistochemistry

Immunohistochemistry was performed as previously described,<sup>29,31,35,55</sup> with primary antibodies specific for ALDH7A1 (rabbit monoclonal antibody IgG #AB53278,

Abcam), ALK (mouse monoclonal IgG3 # M7195, Dako), BCL2L1 (mouse monoclonal IgG2a #AHO0222, Invitrogen), CD8 (monoclonal #NCL-L-CD8-295, Novocastra), DC-LAMP (rat monoclonal IgG2a #DDX0191, Dendritics), FOXP3 (mouse monoclonal IgG #ab450, Abcam), LIPC (mouse monoclonal IgG1, clone XHL1-1C, #sc-21741, Santa Cruz Biotechnology), PAR (mouse monoclonal antibody IgG #AM80, Calbiochem), PDXK (rabbit antiserum #AP7167A, Abgent), PDXP (rabbit antiserum #HPA001099, Atlas Antibodies) and WSB2 (rabbit antiserum #12124-2-AP, Proteintech, Chicago, USA). Staining intensity was quantified on a 0–3 scale, using as a reference the signal observed in fibroblasts or endothelial cells (score 2), while the percentage of positive tumor cells was scored on a 0–100% scale. These variables were integrated into a single score (ranging from 0–300) by calculating the product between staining intensity (0–3) and the percentage of tumor cells (0–100). Staining intensity of DC-LAMP was quantified as the total number of positive cells of 5 regions of interest. Staining intensity of CD8 and FOXP3 were reported as the median density (number of positive cells per  $\mu\text{m}^2$ ) of 5 regions of interest. Positive cells were automatically detected and counted with a custom written macro for ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997–2018.).



**Figure 5.** Spearman's correlation coefficients between immune infiltrate estimates (MCP-counter method) and *ALDH7A1* (A), *BCL2L1* (B) and *LIPC* (C) mRNA levels from different public microarray datasets, of NSCLC, breast cancer, colorectal carcinoma and melanoma (Table 1). Significant correlations are annotated: \* $p < 0.1$ , \*\* $p < 0.05$ , \*\*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ . For each pathology, a combined  $p$  value has been calculated as described in Materials and Methods.

### Expression data in cell lines and immune cell subtypes

We considered two types of cell line expression: (1) expression in a general set of cell lines from The Protein Atlas<sup>56</sup> website (<https://www.proteinatlas.org/>), for which Transcript Per Million (TPM) data are available; (2) expression in immune cell from the Immunological Genome Project<sup>57</sup> (<https://www.immgen.org/>), for which normalized expression (based on DSeq2 package<sup>58</sup>) is available.

### Public microarray data

We used several public microarray datasets for different pathologies: NSCLC<sup>59–61</sup> (and TCGA consortium), breast carcinoma<sup>46,62–65</sup> (and TCGA consortium), colorectal carcinoma<sup>66</sup> (and TCGA consortium, <http://www.intgen.org/>), melanoma.<sup>67–71</sup> We considered only large datasets

(Table 1). R-package MCPcounter was used to estimate immune cell infiltrates from microarray data.<sup>37</sup> For correlation analysis, the Spearman's correlation coefficient and test were employed. We used Fisher's method on one-tail correlation  $p$  values (positive for LIPC, negative for *ALDH7A1*) in order to calculate "combined"  $p$  values for Figure 5 (for the "combined" correlation coefficient, we have chosen the one that has the smallest one-tail  $p$  value).

### Survival analysis

For the analyses depicted in Supplemental Fig. S1, we used the datasets for which clinical data was available: METBARIC,<sup>46</sup> Breast TCGA (TCGA consortium) and Adenoconsortium.<sup>59</sup> We used Cox regression analysis of the R survival package.<sup>72</sup> For the analyses depicted in Supplemental Fig. S2, S3 and S4, we extracted data from the PROGgeneV2 database (dataset



list is given in Supplemental Table S2).<sup>73</sup> We used the R-package meta<sup>74</sup> for the summary analysis and forest plots.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

## Authors contribution

LG provides remunerated consulting to AstraZeneca, OmniSEQ and VL47, and is a member of the Scientific Advisory Committee of OmniSEQ. GK has been holding research contracts with Bayer Healthcare, Genentech, Glaxo Smyth Kline, Institut Mérieux, Lytix Pharma, Nucana, Oncolinx, PharmaMar, Sotio and Tioma and Vasculox. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune.

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